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Development of a highly sensitive ELISA for aldosterone in mouse urine: validation in physiological and pathophysiological states of aldosterone excess and depletion

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[ACTH](#)***

Short running title:

Development of a highly sensitive Aldosterone ELISA

Abstract

Clinical studies have established aldosterone as a critical physiological and pathophysiological factor in salt and water homeostasis, blood pressure control and in heart failure. Genetic and physiological studies of mice are used to model these processes. A sensitive and specific assay for aldosterone is therefore needed to monitor adrenocortical activity in murine studies of renal function and cardiovascular diseases. *Methods:* Antibodies against aldosterone were raised in sheep as previously described. HRP-Donkey-anti-sheep IgG enzyme tracer was produced in our laboratory using the Lightning-Link HRP technique. Aldosterone ELISA protocol was validated and optimised to achieve the best sensitivity. Cross reactivity with likely competing steroids and specificity before and after chromatography were checked. The assay was validated by analysing the urine of mice collected under various experimental conditions designed to stimulate or suppress aldosterone in the presence of other potentially interfering steroid hormones.

Results: Cross-reactivity with major interfering steroids was minimal: corticosterone=0.0028%, cortisol=0.0006%, DOC=0.0048% except for 5 α -dihydro-aldosterone=1.65%. Minimum detection limit of this ELISA was 5.7 pmol/L (1.6 pg/mL). The validity of urinary aldosterone ELISA was confirmed by the excellent correlation between the results obtained before and after solvent extraction and HPLC separation step ($Y=1.048X+0.006$, $R^2=0.998$, $n=42$). Accuracy studies, parallelism and imprecision data were determined and all found to be satisfactory. Using this assay, mean urinary aldosterone levels were (i) approximately 60 fold higher in females than males; (ii) increased 6 fold by dietary sodium restriction; (iii) increased x fold by ACTH infusion; (iv) reduced by >60% in Cyp11b1 null mice.

Conclusion We describe an ELISA for urinary aldosterone which is suitable for repeated non-invasive measurements in mice. Female aldosterone levels are higher than males. Unlike humans, most aldosterone in mouse urine is not conjugated. Increased levels were noted in response to dietary sodium restriction and ACTH treatment. The sensitivity of the assay is sufficient to detect suppressed levels in mouse models of congenital adrenal hyperplasia..

Introduction

Mice are very widely used to study cardiovascular disease. Many genetically modified strains have become available which target all aspects of disease processes including those related to the renin-angiotensin-aldosterone system (Mullins et al). However, a major problem with mice is the limitation of blood volume particularly for repeated measures of analytes which circulate at low concentrations. Also there is the likelihood of introducing stress artefacts when sampling blood in small rodents. These problems are exacerbated for aldosterone where assays must specifically exclude competing steroids such as corticosterone which circulate at 500- 1000 fold greater concentrations. Limitations of volume and stress associated with blood sampling can be avoided by analysing 24h urine samples but there is a clear need for a simple, sensitive and specific method of quantifying aldosterone secretory activity.

The majority of immunoassays use ^3H or ^{125}I labelled tracer d to measure aldosterone levels [1-7]. Although sensitive, the radioactivity is a biohazard and waste disposal is costly and inconvenient. Some non-isotopic assays have been reported [9-16] but reliable and sensitive commercial assays for aldosterone are not widely available and none have been validated for mice. Here we describe an elisa method for the measurement of aldosterone in mouse urine which has the specificity and sensitivity to detect changes in secretory activity in response to pathophysiological conditions of ACTH excess and congenital adrenal hyperplasia as well as physiological differences between males and females and in response to a low sodium diet.

Materials and Methodology

Assay reagents

Anti-aldosterone antibodies were raised in sheep as previously described [5-7] and used at a final dilution of 1:100,000. HRP-Donkey-anti-sheep IgG enzyme conjugate was produced in our laboratory using the Lightning-Link HRP technique following the instructions outlined in the manufacturer's manual (Innova Biosciences, Cambridge) and used to develop the ELISA method at a dilution of 1:2000.

Aldosterone standard was purchased from Sigma-Aldrich, Poole, Dorset, England. All other steroid hormones used for cross-reactivity studies (see Table 1) were obtained from Sigma-Aldrich, Poole, Dorset, UK and Steraloids, Newport, RI, USA. Other reagents: ELISA plates were purchased from Dynex, USA or Griener Bio-One, Germany. β -glucuronidase (from *Helix pomatia*), Tween 20, sulphuric acid, tetra-methyl-benzidine (TMB), hydrogen peroxide (30%) and bovine serum albumin were all obtained from Sigma-Aldrich. Quick-fit glass extraction tubes from Bibby Sterilin Ltd, Staffs, UK Dichloromethane (Analytical grade) and methanol (HPLC grade) were obtained from Fischer Scientific, Leicester, UK.

Solvents and Solutions

Assay buffer: 0.05 mole/L Phosphate Buffer Saline (PBS) pH 7.4 containing 0.1% Bovine Serum Albumin (BSA). Blocking Buffer: 0.025 mole/L PBS pH 7.4 containing 0.5% BSA. Coating buffer 0.025 mole/L PBS pH 7.4. Wash buffer: 0.015 mole/L PBS pH 7.4 containing 0.05% Tween 20. Substrate buffer: 0.2 mole/L Acetate/Citrate buffer pH 4.2. Stop solution: 1 mole/L Sulphuric acid in distilled water. Aldosterone-BSA conjugate purchased from Steraloids, USA dissolved in coating buffer.

Various dilutions of sheep anti-aldosterone were prepared in assay buffer and tested by the ELISA method and an initial dilution of 1:100,000 was found to produce satisfactory signal. Horse Radish Peroxidase-Anti-sheep IgG conjugate solution was prepared in assay buffer and tested at several dilutions and the optimal dilution was found to be 1:2000 of the original conjugate preparation. Substrate solution containing 10mg/mL of TMB in Acetate/citrate buffer pH 4.2 was found to produce maximum colour signal. Standard aldosterone solutions were prepared freshly in assay buffer from a stock aldosterone solution of 1 μM in assay buffer.

Urine samples

Urine samples were collected daily from mice held singly in metabolic cages (Tecniplast, Italy) and stored in universal containers without preservatives at -20°C until required for assay. All animals were adapted to the cages for a minimum 4 days before starting urine collections and were given free access to food and water in a light and temperature controlled room. Unless otherwise stated, mice were adult C57Bl6 (Harlan Olac) fed a diet containing 0.3% sodium (SDS Ltd, Witham, Essex). To test the effects of sodium restriction, urinary aldosterone was measured during adaptation to a diet containing 0.03% sodium. Data showing urinary aldosterone values during ACTH infusion (125ng Synacthen / h) and in Cyp11b1 null mice are from studies which are described elsewhere.

Hydrolysis and extraction protocol of urine samples

Steroids in 0.5ml urine were extracted into organic solvent by vortexing with 10 mL dichloromethane in glass-stoppered tubes and the extract washed successively with 1 mL aliquots of 0.05M NaOH, 0.05M HCl and distilled water. The solvent was then evaporated at 40°C and the residue reconstituted in 1mL assay buffer. Extraction recovery was checked in 20 urine samples by using ³H-Aldosterone (Amersham) and found to be 95.2 % ± 5.6 (SD); results were not corrected for recovery. Steroid conjugates in urine (0.5ml) were hydrolysed by incubating with 0.5ml β-glucuronidase solution (*Helix Pomatia* 10,000 units/ml in 0.5 M acetate buffer pH 5.5) at 37°C for 18-24h. Aldosterone values were not significantly affected by hydrolysis.

Optimisation of Assay protocol for the Urinary aldosterone ELISA

Aldosterone was measured using an indirect ELISA technique, with some modifications to sensitise the assay [27-29]. The ELISA standard curve was optimised for the amount of conjugate needed to coat the plate, time and temperature of incubation and the dilution of aldosterone antibody required. All samples were assayed in duplicate. 96 well ELISA plates were coated with aldosterone-3-CMO-BSA conjugate (200μL coating buffer containing 0.2 μg conjugate/mL). The plates were then covered with film and left overnight at 4°C. The coating solution was discarded, plates were washed four times with 250 μl wash buffer and then blocked by incubating with 200 μl blocking buffer for 1h at 37°C. The blocking buffer was discarded and plates were blotted and left to dry in air. Aldosterone standards (0-100 nM) and urine extracts (50μl in duplicate) were added to the wells followed by 100 μl of antibody solution. After incubation at room temperature for two hours, the contents were discarded and the plate washed with buffer as before. At this point 100 μl of HRP-anti-sheep enzyme conjugate was added to each well and the plates incubated at room temperature for another hour. Plates were emptied and washed before adding 100ul substrate solution. Finally, after a 15minute incubation at room temperature, 50 μl of stop solution was added and absorbance was read at 450nm in an MRX plate reader.

Calculation of results

Aldosterone concentration in the sample was calculated by using the semi-log cubic spline software data reduction method built in to the MRX ELISA reader. After taking account of urine volume and body weight, values are then expressed as pmol/day/g body weight ± SE.

HPLC system procedure

Specificity was checked by comparing results with and without HPLC separation. Dichloromethane extracts of 42 urine samples were divided into two aliquots. Both aliquots were evaporated to dryness, one was reconstituted in assay buffer and assayed directly for aldosterone and the other was reconstituted in HPLC mobile phase. (water:isopropanol, 87:13, v/v). Samples were fractionated using a Dionex HPLC system with a reverse phase Sunfire C18 15cm-column at a temperature of 35°C, a flow rate of 1ml/min and with a mobile phase containing stepped increases in the proportion of isopropanol in the mobile phase (13% x 6 min, 15% x 16 min; 21% x 8 min; 28% x 8 min; 39% x 6min). Retention times of 9.9, 20.4, 28.9 and 36.6 minutes for authentic aldosterone, corticosterone, deoxycorticosterone and progesterone standards were noted by monitoring eluate at 245nm. Corresponding fractions for urine samples were collected, dried down, and reconstituted in ELISA assay buffer.

Validation of aldosterone ELISA

Cross-reactivity of various steroid hormones with the aldosterone antibody using the optimised ELISA standard curve was determined according to the methods of Abraham [32]. Validation procedures were carried out as previously published [27,28]. Accuracy was determined by adding known amounts of standard aldosterone (0.05 - 10.0 nmol/L) to urine samples. All samples (spiked and non-spiked urine) were assayed by aldosterone ELISA. The amount of aldosterone recovered was then calculated by subtracting the spiked dose from the value obtained for each spiked urine sample. Imprecision was estimated by replicate determinations of aldosterone levels in low, medium and high pools measured in the same (intra-) or different (inter-) assays over a period of 20 weeks. Blank values of the aldosterone ELISA were checked by using distilled water instead of the urine sample and the results were not statistically different from the zero standard blank of the ELISA reader (0.042 ± 0.015). To test for parallelism and the effect of varying urine osmolality on the assay, serial dilutions of 4 urine samples taken from different animals were made in distilled water and the dilutions were extracted and treated as described above, then assayed.

Correlation studies

Various urine samples obtained from several animals were assayed for urinary aldosterone excretion after enzyme hydrolysis and solvent extraction by the ELISA method and by an in-house RIA method (n=33). To further verify the results of the aldosterone ELISA, 42 urine samples were also assayed before and after an HPLC separation step as described above. Linear regression analysis was used throughout to compare the results obtained by different methods.

Results

Optimisation of aldosterone standard curve The effect of varying the amount of aldosterone-BSA conjugate on the standard curve is shown in figure 1. Typical standard curve data for aldosterone ELISA are shown in Figure 2. Sensitivity (minimum detection limit) for the aldosterone ELISA standard curve was determined according to the method of Abraham [33]. The amount of aldosterone that produced a statistically significant drop

in signal compared with that of the zero standard was found to be 5.2 pM or 1.5 pg/mL or 75fg/well. When the limits of detection (LOD) was determined according to IUPAC as the mass of analyte required to decrease the signal of zero standard by 2 SD [30], the sensitivity was 12.5 pmole/L or 3.6 pg/mL or 180fg/well.

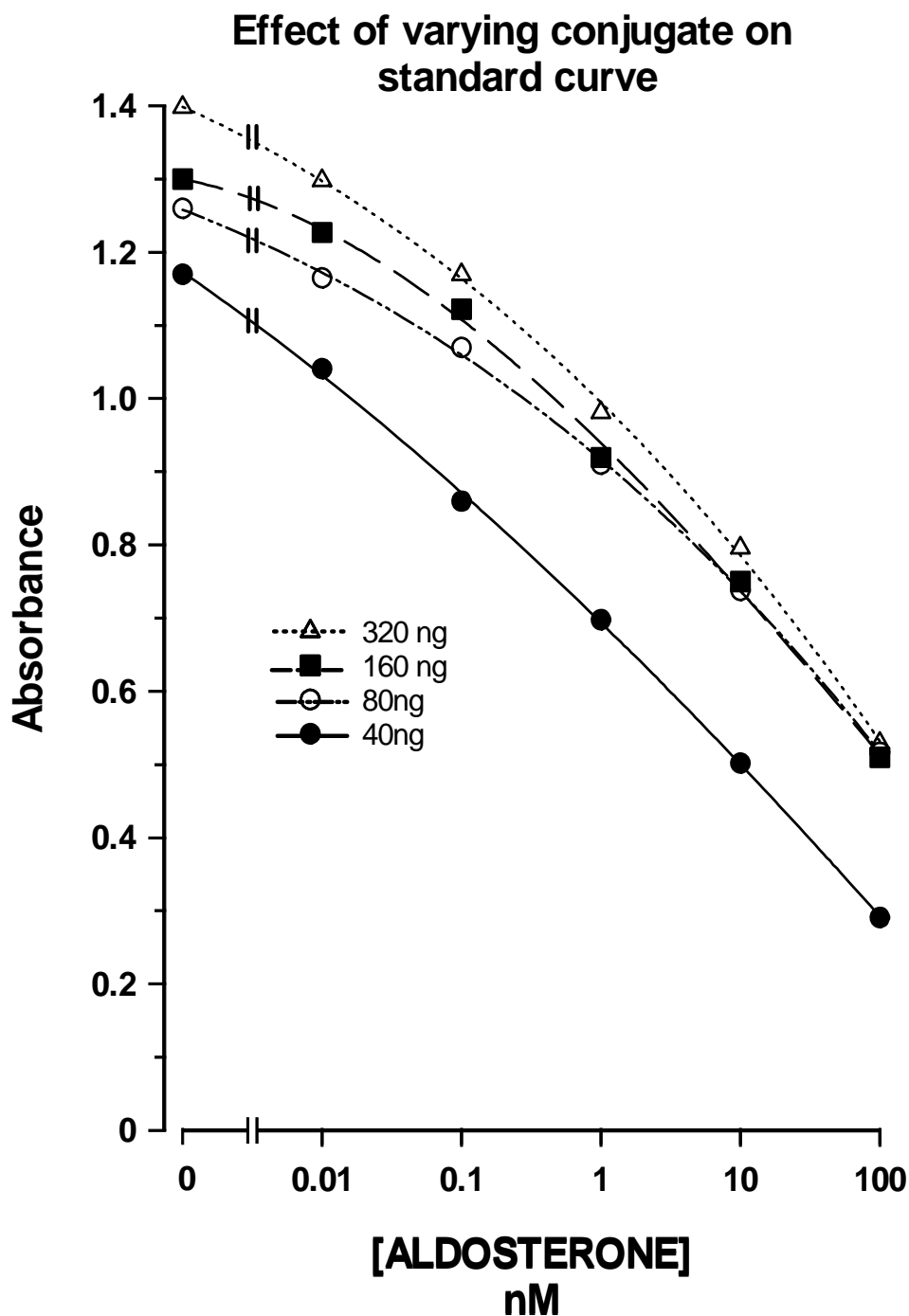


Figure 1. Effect of varying the amount of aldosterone conjugate used to coat the plate on the ELISA standard curve. Values range from 10 μ L to 80 μ L/mL (0.2-1.6 μ g/mL) of conjugate solution.

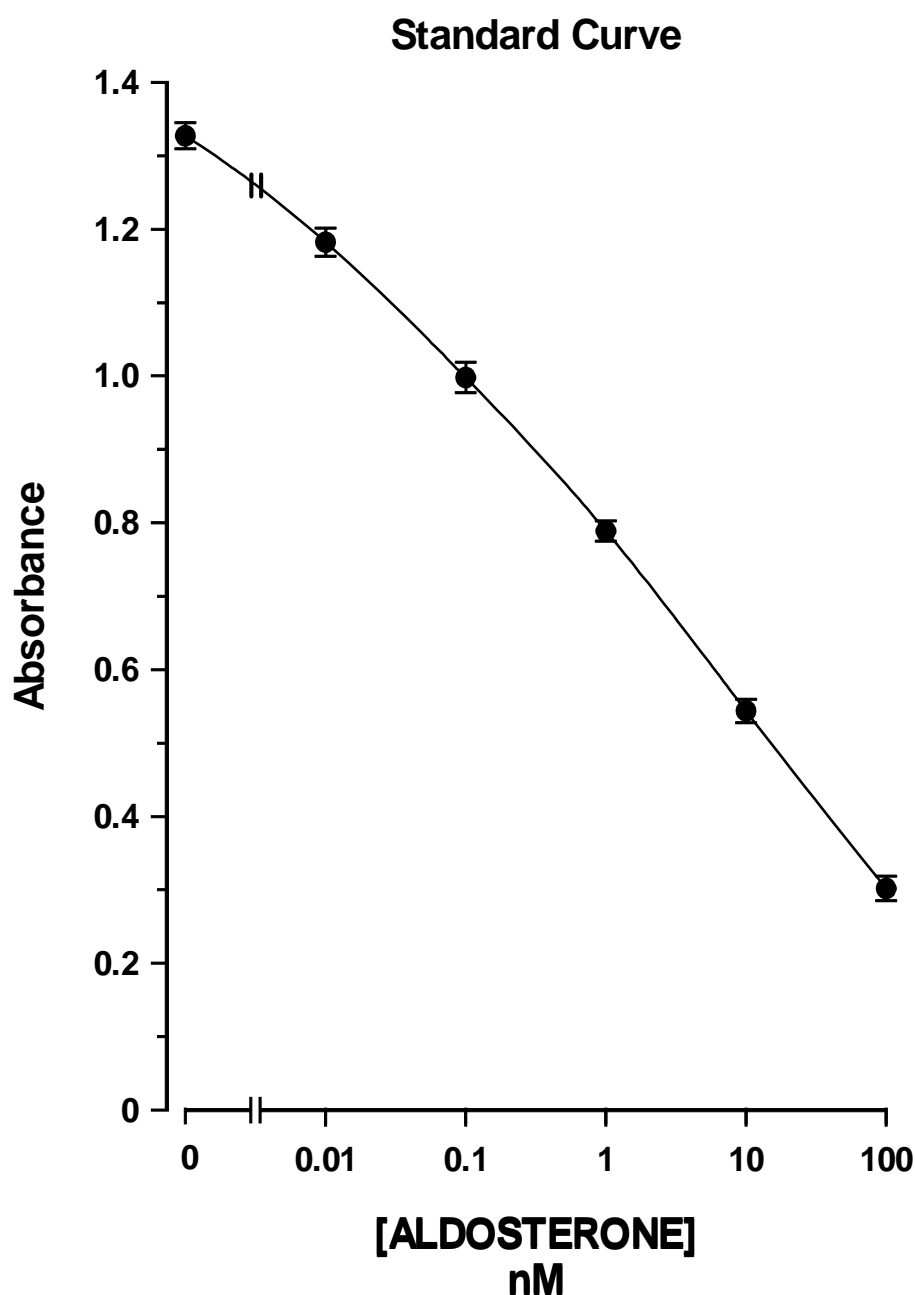


Figure 2, Typical aldosterone ELISA standard curve (mean \pm SE; n = 16).

Validation of aldosterone ELISA

Cross-reactivity of aldosterone antibody with major interfering steroids was minimal except for 5α -Dihydroaldosterone = 1.65% (see Table 1). Accuracy studies, parallelism and imprecision data were determined and all found to be satisfactory. The accuracy of aldosterone ELISA is shown in Table 2 (recovery of added aldosterone ranged from 98.2-106.1% with a mean of 100.6% \pm 3.66). The results of serial dilution of 4 urine samples were shown in Table 3 (%CV for calculated results ranged from 2.2-3.5%). The results of

intra-assay and inter-assay imprecision for 3 urine pools ranged from 5.12- 8.74% CV and 5.78- 10.07% CV respectively (see Table 4).

Table 1: Cross-reactivity data for the sheep aldosterone antibody used in the ELISA

Steroid	% Cross Reactivity
Aldosterone	100.00
5 β -Dihydroaldosterone	0.045
5 α -Dihydroaldosterone	1.65
Tetra hydroaldosterone	0.0018
Corticosterone	0.0028
Deoxycorticosterone	0.0048
Cortisol	0.0006
11-Deoxycortisol	0.0001
Progesterone	0.0011
17 α -OH-progesterone	0.0007
18-OH-Deoxycorticosterone	0.002
18-OH-corticosterone	0.0006
20 α -OH-progesterone	0.0003
Tetrahydrodeoxycorticosterone	0.0002
5 β –Tetrahydrocorticosterone	< 0.0001
5 α -Dihydrocorticosterone	0.0007
5 β –Dihydrocortisol	< 0.0001
5 α –Dihydropregnenolone	0.0008
Testosterone	0.0006
Androstenedione	< 0.0001
Dehydroepiandrosterone	< 0.0001
Cholesterol	< 0.0001
Estrone	< 0.0001
Estradiol-17 β	< 0.0001
Estriol	< 0.0001

Table 2, Accuracy data obtained by the aldosterone urine ELISA. Percent recovery of aldosterone standard added to urine samples (n = 9).

Amount added (nmole/L)	Mean Amount recovered (nmole/L)	% Mean Recovery
0.05	0.0512	102.4
0.10	0.106	106.1
1.25	1.23 (n= 9)	98.2
2.5	2.48 (n= 9)	99.4
10.0	9.7 (n= 9)	97.0
Overall Mean \pm 1SD		100.6 \pm 3.7

Table 3, Results of urinary aldosterone samples obtained by the serial dilution of 4 urine samples.

Sample No.	1	2	3	4
Mouse genotype	Wild (+/+)	Wild (+/+)	KO (-/-)	KO (-/-)
Calculated value	(nmole/day)	(nmole/day)	(nmole/day)	(nmole/day)
Neat Value	9.878	12.576	0.426	0.886
Dilution factor				
X2	9.824	12.724	0.432	0.912
X4	10.112	12.048	0.456	0.856
X8	10.224	12.516	0.466	0.924
X16	9.685	12.192	0.446	0.934
Mean	9.9446	12.5596	0.4492	0.9024
±1SD	0.219351	0.296082	0.019005	0.03154
% C.V.	2.20%	2.36%	4.23	3.5

Table 4, Imprecision data for the urinary aldosterone ELISA.

a) Intra-assay imprecision

Aldosterone Mean (nmole/day)	± 1SD	%C.V. (Coefficient of Variation)
24.944	1.277	5.12
1.722	0.128	7.43
0.286	0.025	8.74

b) Inter-assay imprecision

Aldosterone Mean (nmole/day)	± 1SD	%C.V. (Coefficient of Variation)
24.689	1.428	5.78
1.756	0.135	7.69
0.288	0.029	10.07

Comparison with RIA and the effects of HPLC

Comparison of the results by the present ELISA with those estimated by the in-house RIA method is shown in Fig. 3 ($\text{ELISA} = 0.657\text{RIA} - 44.418$, $R^2 = 0.897$, $p < 0.001$, $n = 33$). The values obtained by the aldosterone ELISA were 20-35% lower than those measured by the RIA.

To confirm the specificity of the assay, results were compared with and without HPLC separation. There was an excellent correlation between the two sets of values (figure 4) $\text{non HPLC} = 1.046(\text{HPLC values}) + 0.0026$, $r^2 = 0.998$, $p < 0.001$, $n = 42$

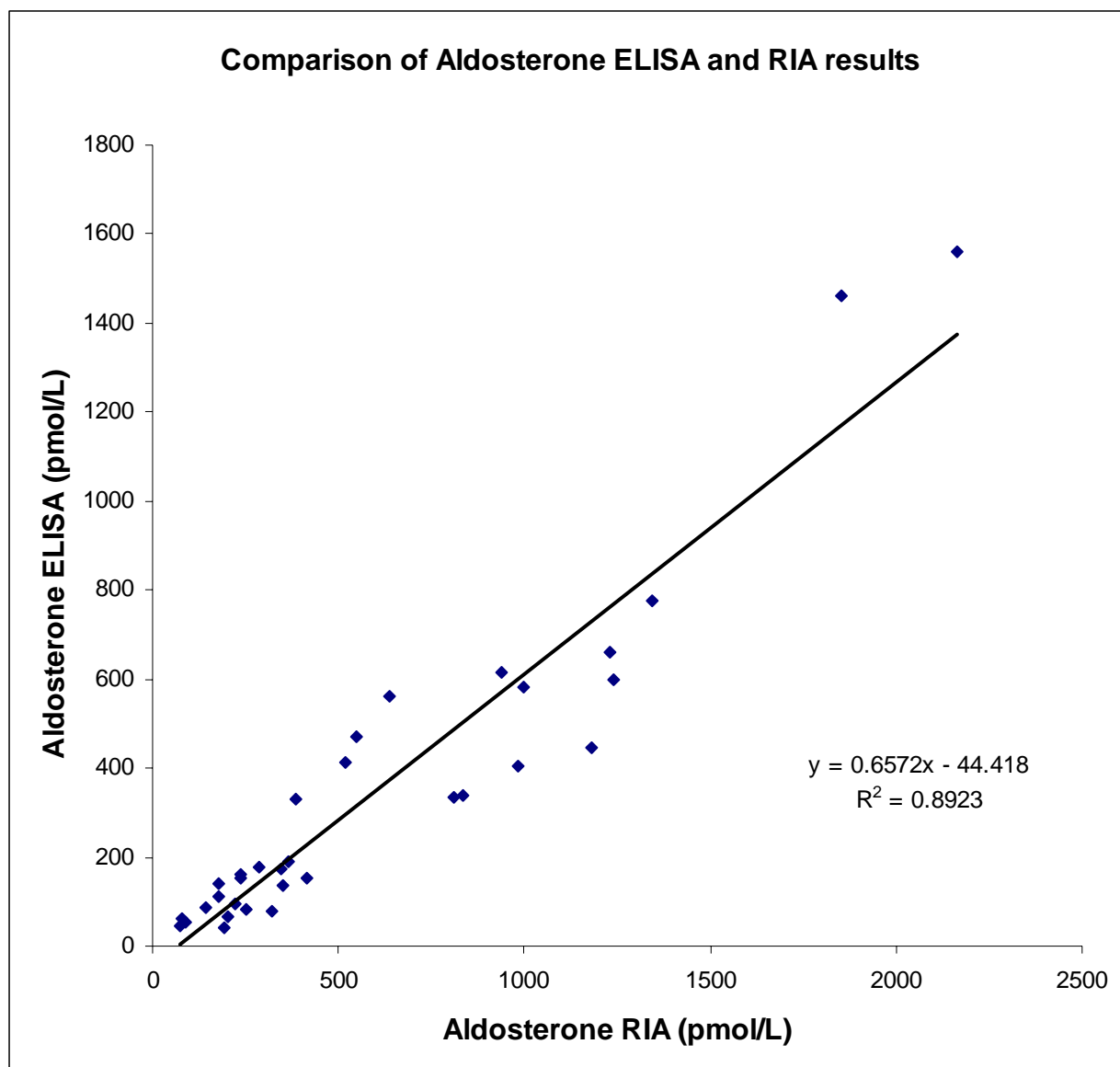


Figure 3, Comparison of urinary aldosterone results obtained by the new ELISA with those of the radioimmunoassay.

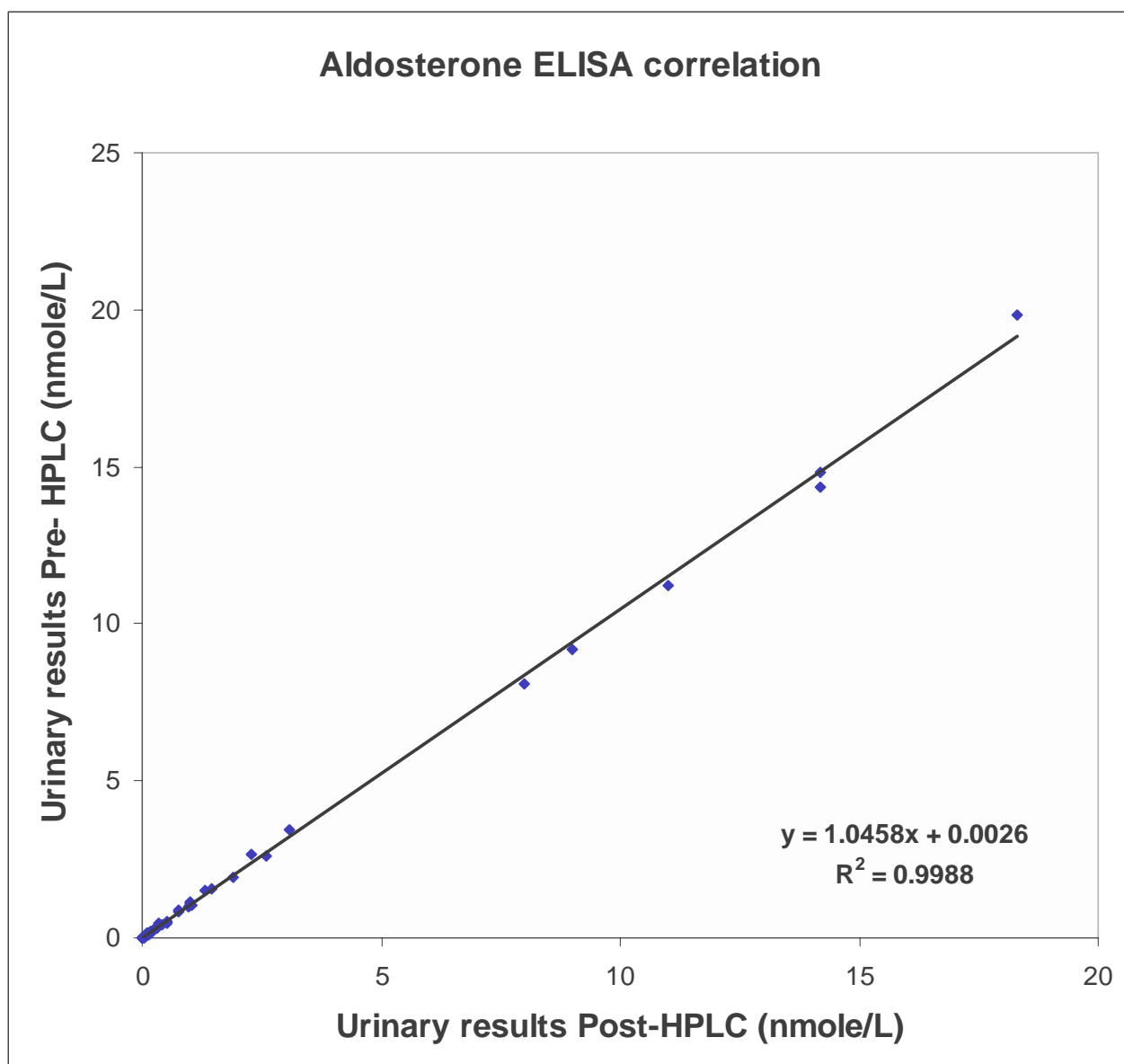


Figure 4, Comparison of urinary aldosterone results before and after HPLC fractionation.

Urinary aldosterone animal data

Figure 5 shows aldosterone levels in normal male and female mice and in a mouse model of congenital adrenal hyperplasia. Female mice levels were approximately 60 fold higher than those of male mice. Urinary aldosterone values in Cyp11b1 null mice were 60-80% lower than wild type controls. Cyp11 b1 null mice have suppressed aldosterone values because they secrete 25-65 times higher amounts of deoxycorticosterone; an alternative mineralocorticoid.

Figure 6 shows the effects of dietary sodium restriction. Urinary aldosterone of normal male type mice increased more than 6 fold over the first 4 days of adaptation from a normal (0.3% NaCl) to a low sodium (0.03% NaCl) diet.

Figure 7 shows the infusing ACTH in normal mice on urinary aldosterone values. Aldosterone is markedly increased during the first few days of infusion but reduces to

near pre-infusion levels during the second week. In contrast, corticosterone values which are also stimulated >100 fold by ACTH (data not shown) are higher in the second week than the first week. This highlights the fact that even under pathophysiological conditions, excessively high glucocorticoid values do not interfere in the measurement of aldosterone.

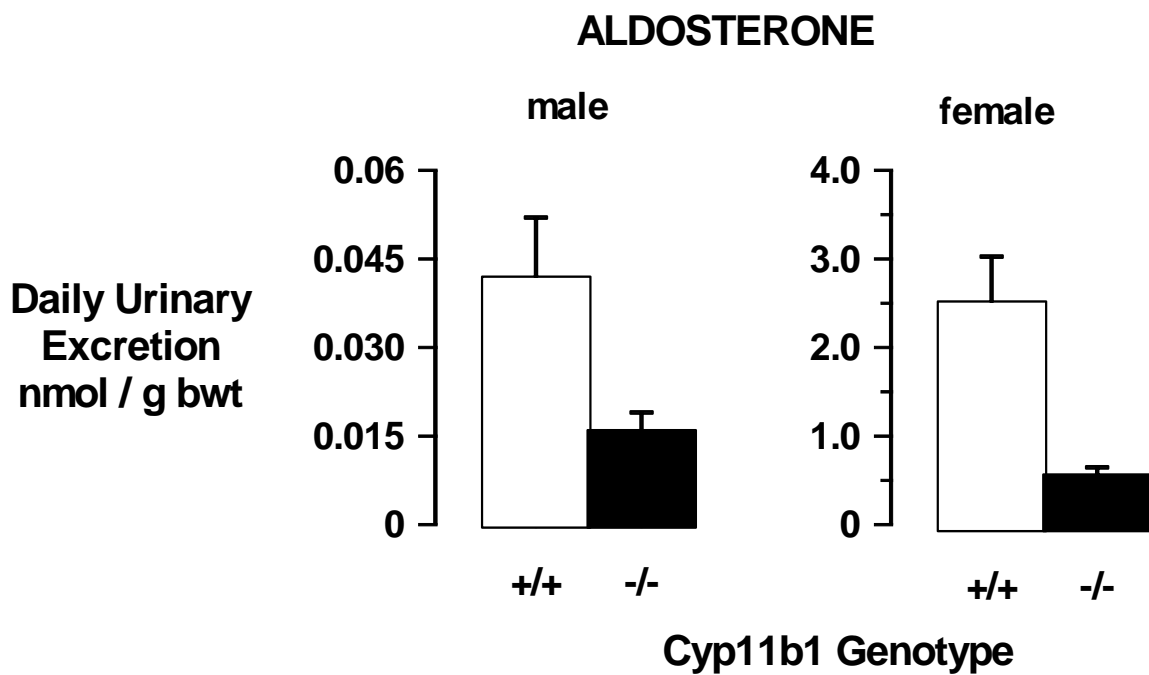


Figure 5: Aldosterone daily urinary excretion in male and female animals. Wild type (+/+); Cyp11b1 knockout mice (-/-) (data from Mullins et al, JBC in press)

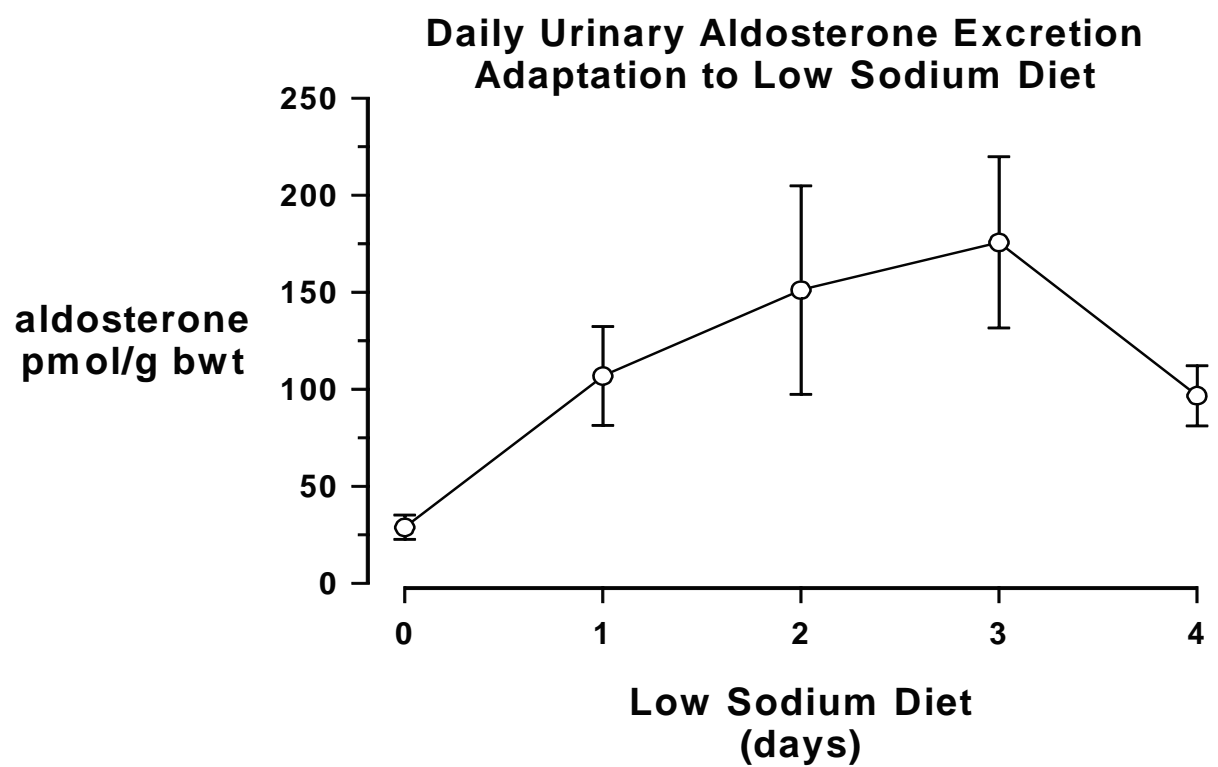


Figure 6: Effect of low sodium diet and adaptation on urinary aldosterone excretion in male mice as measured by the present ELISA.

Figure 7 Effect of Synacthen infusion (3µg/d) via Alzet mini-osmotic infusion pumps on urinary aldosterone excretion in male mice (n=5, ± sem). (data are from unpublished work of Dunbar et al)

Discussion and Conclusion

A highly sensitive, specific and easy to perform ELISA has been developed and validated for the measurement of aldosterone in mouse urine samples. Estimation of 24 hour urinary aldosterone values represents the ideal medium to investigate the physiology and pathophysiology of mouse models in which adrenocortical function is affected. It is impractical to collect repeated blood samples from these animals because blood volume is too small to permit sampling at more than one time point and because there are risks of introducing artefacts from stress associated with handling the animal or from anaesthesia used to collect un-stressed samples. We have established that the assay is sufficiently sensitive to identify normally low levels of aldosterone and even abnormally low levels when secretion is suppressed. The specificity of the assay has been confirmed by in vitro crossreactivity tests and by demonstrating appropriate responses to a variety of physiological and pathophysiological tests.

Specificity is a particular problem when measuring urinary aldosterone firstly because it circulates at much lower concentrations than other corticosteroid hormones with similar chemical structures and, secondly, because urine contains relatively high levels of aldosterone metabolites. In vitro tests confirmed that the antibody discriminated aldosterone from other likely competitors. Only dihydroaldosterone came close to being a problem. Previous studies have found erroneously high values for aldosterone when antibodies with greater affinity for this reduced metabolite were used but not with a different antibody with lesser affinity. It is notable that the specificity of this alternative successful antibody was less than the one used in the present ELISA. Using samples with a wide range of aldosterone concentrations, we have shown that values measured before and after HPLC fractionation are in agreement. We have also tested the assay with samples collected from mice treated longterm with an infusion of ACTH. This treatment exaggerated the normal disparity between corticosterone and aldosterone yet we were able to demonstrate the transient and much lower increase in urinary aldosterone despite sustained high corticosterone levels.

Although ACTH is an established modulator of aldosterone secretion, the primary regulators are circulating angiotensin II and potassium concentrations. Dietary sodium controls renin-angiotensin which regulates aldosterone secretion to maintain salt and water homeostasis (Williams 2004). We therefore measured urinary aldosterone during adaptation to low sodium food as a test of the assay's ability to detect physiologically relevant changes. The results show an aldosterone response to switching from a 0.3 to a 0.03% sodium after 1 day reaching a maximal 6 fold increase after three days. Furthermore we found that under normal dietary conditions, female urine has 60-fold higher concentrations than that of males. In part this also reflects higher circulating levels of the renin-angiotensin system. However, it is also known that the female adrenal gland is larger than that of males and that the renin-angiotensin-aldosterone

system is different in male and female rodents (Deschepper et al, Inagami). In particular, Morris and colleagues () have demonstrated that following an injection of aldosterone, the clearance rates of aldosterone and the nature of urinary and fecal aldosterone metabolites is different in males and females.

Due to the use of highly specific antibodies and careful optimization of the amount of steroid conjugate adsorbed onto the plates, the new ELISA was sensitive enough to detect abnormally low levels. In Cyp11b1 null mice, adrenal 11 β hydroxylase activity (the final enzyme the synthesis of glucocorticoid hormones) is deficient. This leads to an accumulation of a steroid substrate, deoxycorticosterone with mineralocorticoid properties that suppress the renin-angiotensin system. Using the present ELISA we showed that urinary aldosterone was suppressed by 60-80% in male and female samples from homozygous Cyp11b1 null mice when urinary deoxycorticosterone was increased 30-60 fold. Our expectation is that this assay will be particularly useful in phenotyping other pathophysiological models of low aldosterone (eg 11hsd2 null mice) as well as adaptation to high sodium diet.

The assay has been validated for accuracy, parallelism (dilution of urine samples) and imprecision with samples from wild type, heterozygote and cyp11B1 null mice. The results obtained were found to be very satisfactory. Furthermore, the validity of the aldosterone urinary ELISA was confirmed by the excellent correlation between the results obtained by the assay before and after an HPLC fractionation step. The results of the ELISA correlate well with our in-house RIA methods using tritiated-aldosterone label. However values by the new ELISA were (20-35%) lower than those by RIA. This is probably due to the ^3H -aldosterone tracer that has previously been shown to produce inferior specificity and imprecision data [7]. The ELISA is at least as fast as RIA and obviates the need for expensive and hazardous radioactive tracer thereby reducing the costs of raw materials and their disposal. The assay is ideal for use in large projects involving huge number of samples.

In humans, aldosterone is mainly excreted as a glucuronide in urine (Mohring et al, Tait et al) and thus estimation of total aldosterone is usually done after hydrolysis of the conjugate [25, 26]. We have used the enzymatic hydrolysis method (β -glucuronidase, *Helix pomatia*, Sigma) as optimised by Ferchaud *et al* [25, 35] to pre-treat mouse urine. Although values of other adrenocorticosteroids (eg deoxycorticosterone) were increased by hydrolysis particularly in female samples, aldosterone values were not significantly affected. This would suggest that prior to glucuronidation, aldosterone is metabolised to a compound which is not recognised by the ELISA and, possibly, that the renal enzyme responsible for the production of human urinary aldosterone glucuronide may not necessarily be expressed in mouse kidney.

In conclusion, a simple and highly sensitive ELISA has been developed to estimate urinary excretion of aldosterone. The assay can identify physiological responses to dietary sodium intake in mice and has demonstrated differences in male and female values. The assay has also demonstrated its usefulness in analysing high and low levels in pathophysiological states of excess ACTH and congenital adrenal hyperplasia.

List of Abbreviations

BSA = Bovine Serum Albumin

ELISA = Enzyme linked immunosorbent assay

C.V. = Coefficient of variation

CYP11b1 = Gene encoding 11 β -Hydroxylase enzyme

GC/MS = Gas Chromatography/Mass Spectrometry

HPLC = High Performance Liquid Chromatography

HRP = Horse Radish Peroxidase

IgG = Immunoglobulin

LOD = Limit of Detection

PBS = Phosphate Buffered saline

RIA = Radioimmunoassay

SD = Standard deviation

TMB = Tetra-Methyl Benzidine

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